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Original Article

Enteric helminth-induced type-I interferon signalling protects against pulmonary virus infection through interaction with the microbiota

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Abstract

Background: Helminth parasites have been reported to have beneficial immune modulatory effects in allergic and autoimmune conditions and detrimental consequences in tuberculosis and some viral infections. Their role in co-infection with respiratory viruses is not clear.

Objective: Here, we investigated the effects of strictly enteric helminth infection with *Heligmosomoides polygyrus* on respiratory syncytial virus (RSV) infection in a mouse model.

Methods: A murine helminth/ RSV co-infection model was developed. Mice were infected by oral gavage with 200 stage 3 *H. polygyrus* larvae. 10 days later, mice were infected with either RSV or UV-inactivated RSV (UV-RSV) intranasally.

Results: *H. polygyrus* infected mice showed significantly less disease and pulmonary inflammation after RSV infection, associated with reduced viral load. Adaptive immune responses including Th2 responses were not essential since protection against RSV was maintained in *RAG1*$^{-/-}$ and *IL-4Rα*$^{-/-}$ mice. Importantly, *H. polygyrus* infection upregulated expression of type I IFNs and IFN stimulated genes (ISG) in both the duodenum and the lung, and its protective effects were lost in both *IFNAR1*$^{-/-}$ and germ-free mice, revealing essential roles for type I IFN signalling and microbiota in *H. polygyrus* induced protection against RSV.

Conclusion: These data demonstrate that a strictly enteric helminth infection can have remote protective antiviral effects in the lung through induction of a microbiota-dependent type I IFN response.

Key Messages

- Strictly enteric helminth infection induces type I IFN production and ISG expression in both the duodenum and the lung.
- Helminth-induced type I IFN signalling and the presence of the microbiota are critical for protection against RSV infection.

Capsule Summary

Strictly enteric helminth infection protects against RSV-infection through microbiota-dependent induction of type I interferon in the lung, a novel mechanism which in the future may reveal new targets for the prevention and treatment of RSV infection.

Key Words

RSV; helminths; *Heligmosomoides polygyrus*; type I interferon; microbiome.

Abbreviations used

cDC: Conventional DC

ES: excretory secretory

HES: *H. polygyrus* ES

ISG: IFN stimulated gene

88 LRTI: lower respiratory tract infection

89 PVM: pneumovirus of mice

90 PRR: pathogen recognition receptor

91 OAS: 2’ 5’ oligoadenylate synthetase

92 RSV: respiratory syncytial virus

93

INTRODUCTION

Respiratory syncytial virus (RSV) is a major respiratory pathogen. It infects nearly all infants by the age of 2 years (1), but does not induce lasting immunity and leads to recurrent infections throughout life. It is estimated that worldwide, 33.4 million children under the age of 5 experience RSV lower respiratory tract infection (LRTI) annually and 10% of these require hospitalisation, resulting in up to 199,000 deaths (2-4). There is also major morbidity and mortality due to RSV in the elderly (5). Currently, there is no effective vaccine available for RSV, and treatment is limited to supportive care. Severe RSV LRTI is associated with and thought to be due to severe pulmonary inflammation.

In addition, severe RSV infection during infancy has also been associated with increased risk for asthma development. There is substantial evidence indicating that children hospitalized with RSV-bronchiolitis, are more likely to experience recurrent wheezing episodes for a prolonged period of time after recovery from this illness (6-9).

Helminths infect approximately 3 billion people worldwide. It has long been proposed that infection with helminths could suppress the development of immune-mediated disease, as in countries where their prevalence is high the prevalence of asthma, allergy, and autoimmune conditions has been found to be correspondingly low (10). Intestinal helminths in particular have been of major interest due to their ability to modulate host immune and inflammatory responses to foreign antigens (11-16) and several clinical trials have been carried out or are underway, assessing their utility as therapeutic agents in inflammatory bowel disease, multiple sclerosis and asthma (17).

Helminth infections rarely occur in isolation and co-infections are very common with varying effects such as reduced pathogen control and increased disease, as reported for HIV infection

and tuberculosis (18-21). Recent experimental models in mice report reactivation of systemic latent γ-herpesvirus and reduced control of enteric norovirus replication (22, 23) indicating that in these systems, helminth infection suppresses anti-viral immunity resulting in increased viral replication. However, the impact of helminth infection on respiratory viruses is not well understood. Clinical data is lacking, but mouse models suggest reduced influenza-induced pathology in helminth co-infection (24, 25).

Here, we investigated whether infection with the strictly enteric murine helminth *Heligmosomoides polygyrus* would change the course of disease and inflammation during RSV infection. This study demonstrates protective effects of helminth infection on RSV infection and reveals a novel mechanism of type I IFN induction by enteric helminth infection at a site distant from the gut.

METHODS

Animals

BALB/c, C57BL/6, IL-4Rα-/-(79), RAG1-/-(80), IL-33R-/- (BALB/c background), IFNAR1-/- (81) and Camp-/- (82) (bred to congenicity on a C57BL/6J Ola Hsd background) mice were bred in-house at the University of Edinburgh. Germ-free BALB/c mice were obtained from the Clean Mouse Facility (CMF), University of Bern, Bern, Switzerland, and were compared to SPF BALB/c mice from Charles River Breeding Laboratories (l'Arbresle Cedex, France). 6-12 week old female mice were infected by oral gavage with 200 stage 3 H. polygyrus larvae. Ten days later, mice were intranasally infected with RSV or mock infected with UV-inactivated RSV (UV-RSV) (standard coinfection protocol).

Parasites, parasite products and virus stocks

Parasites were maintained as previously described (83). Stage 3 H. polygyrus larvae were irradiated with 100, 200 or 300 Gy using a GSR-C1 irradiator at a rate of 6.2 Gy/min prior to administration by oral gavage. Axenic H. polygyrus larvae were produced as previously described (84). Plaque purified human RSV (Strain A2, ATCC, United States) was grown in Hep-2 cells as previously described (40).

Whole body plethysmography

Baseline respiratory effort was assessed in individual mice, using whole body plethysmography (Buxco Europe, UK). Mice were placed into individual chambers, and baseline measurements were recorded for 5 minutes. Enhanced pause (Penh) values were recorded, averaged, and expressed as absolute values as previously described (85).

RSV immunoplaque assay

RSV titres were assessed as previously described (27), in lung homogenate by titration on HEp-2 cell monolayers in 96-well, flat-bottom plates. Twenty-four hours after infection, monolayers were washed, fixed with methanol, and incubated with peroxidase-conjugated goat anti-RSV antibody (Biogenesis, United Kingdom). Infected cells were detected using 3-amino-9-ethylcarbazole and infectious units enumerated by light microscopy.

Lung cell isolation and flow cytometry

Right lung lobes were excised, cut into small pieces, incubated on a shaker with collagenase A (Sigma; 0.23 mg/ml PBS) at 37 °C for 45 minutes and sheared through a 19 gauge needle. After red blood cell lysis (Sigma), the single cell suspension was passed through a 40 μm cell strainer and stained using viability dye eFluor 780 (eBioscience, Hatfield, UK). The following anti-mouse antibodies were used to phenotype lung immune cells: PDCA-1 (EBIO-927), Ly6G (RB6-C5), NKp46 (29A1.4), B220 (RA3-6B2) eFluor 450 conjugated (eBioscience), Ly6C (AL-21), CD8 (Ly-2) Fluorescein isothiocyanate (FITC) conjugated (BD Bioscience), CD11b (M1/70), CD4 (RM4-5) Phycoerythrin (PE) conjugated (eBioscience), CD45 (30-F11) eFluor605 Nanocrystal (NC605) conjugated (eBioscience), CD49B (DX5), CD19 (6D5) (Biolegend), F480 (Cl:A3-1) AlexaFluor 647 conjugated (AbD

Serotech) , MHCII (M5/114.15.2), CD3 (145-2C11) PercpCy5.5 conjugated (Biolegend), CD19 (EBIO1D3), CD3 (17A2) (eBioscience), Ly6G (1AB) AlexaFluor 700 conjugated (BD Bioscience) CD11c (N418) PE-Cy7 conjugated (eBioscience). Isotype control antibodies were used on pooled samples. Cells were gated as viable and CD45+ and subsequently phenotyped based on their markers as follows: Ly6G- CD19- CD3- CD49B+ NKp46+ NK cells, Ly6G- CD19- CD3- MHCII+ CD11B+ CD11C+ conventional dendritic cells, Ly6G- CD19- CD3+ CD4+ or CD8+ T cells, Ly6G- CD3- CD19+ CD19+ B220+ B cells. Samples were collected using LSR Fortessa II. Post-acquisition analysis performed using FlowJo version 7.6.5 software (treestar.inc, Oregon, USA).

Real Time PCR

Lung and duodenum was harvested and homogenised in 1 ml of TRIzol (Invitrogen) using a TissueLyser. Complementary DNA (cDNA) was made from the extracted RNA using the Qiagen QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. 1 µg RNA was used for the reverse transcription. Primers were diluted in TE buffer to a final concentration of 0.025 nM/µl and probes to 0.005 nM/µl. Custom primers and probes were purchased from Jena Bioscience or Applied Biosystems. PCR amplification was carried out in a 25 µl volume made up of custom 7 µl primer probe mix (300nM primers and 200nM probe), 12.5 µl TaqMan mastermix (Applied Biosystems); 1.75 µl H20; 1.25 µl 18S (Applied Biosystems); 2.5 µl DNA template. 1.25 µl of pre-made primer probe mix was used in the following mixture: 12.5 µl mastermix; 5 µl H20; 1.25 µl 18S; 2.5 µl DNA template. IFN-β (Mm00439552_s1) and Camp (Mm00438285) primers and probes were bought premade from Life Technologies. Custom primers used are shown in Table I.

195 **ELISA**

196 IFN-α and IFN-β was measured using an ELISA kit (PBL, Interferon Source) according to
197 the manufacturer's instructions.

198

199 **Statistical Analysis**

200 All data were analysed using Prism 6 (Graphpad, La Jolla, CA, USA). Analysis of 2 groups
201 used an unpaired t-test. Analysis of 3 or more groups was either using One-way ANOVA
202 with Tukey's or Bonferroni's post test or Two-way ANOVA with Bonferonni's post test.
203 Unless otherwise stated, the differences are non-significant. *** $p < 0.001$; ** $p < 0.01$; * p
204 < 0.05. Outliers were tested for using Grubb's test, and removed if determined to be an
205 outlier.

206

207 **Study Approval**

208 All procedures were carried out with institutional ethical approval and under Home Office
209 licences. Germ-free animal experiments were performed according to institutional guidelines
210 and to Swiss Federal and Cantonal laws on animal protection.

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RESULTS

H. polygyrus protects against RSV disease and inflammation and reduces viral load.

Mice were infected with *H. polygyrus*, and 10 days later, when adult worms emerge into the lumen of the gut, mice were infected with RSV. *H. polygyrus* co-infection protected against RSV-induced weight loss (see Figure 1A) and reduced RSV-induced increases in enhanced pause, which are indicative of deterioration in baseline respiratory effort (see Figure 1B).

RSV infection in the mouse model induces pulmonary inflammation with cellular infiltration, specifically of NK cells, $CD8^+$ T cells and conventional DCs (cDC) (26, 27). In mice co-infected with *H. polygyrus*, RSV-induced increases in NK cell, B cell (see Figure E1A and E1B in the Online Repository) and $CD8^+$ T cell numbers were absent (see Figure 1C) while the increase in cDC numbers was significantly reduced (see Figure 1D). Early pro-inflammatory cytokine production of IL-6 and TNF-α on day 2 was induced to a significantly lower level in *H. polygyrus* infected mice compared to those infected with RSV alone (see Figure E1C and E1D in the Online Repository). IFN-γ increased with RSV infection, but was not significantly suppressed in co-infected mice, indicating selective inhibition of a pathway independent of IFN-γ (see Figure E1E in the Online Repository).

Given these changes in RSV-induced signs of disease, we asked whether *H. polygyrus* suppresses the immune response or directly alters magnitude of RSV infection. Lung RSV titres, assessed by plaque assay, were reduced following *H. polygyrus* infection, without changes in the kinetics of replication (see Figure 1E). In C57BL/6 mice *ex-vivo* plaque assays for RSV are unreliable due to low viral load (28), therefore we tested the effects of co-infection in C57BL/6 mice by measuring expression of the RSV L gene in the lung by

RTPCR, as an indicator of viral load. L gene expression was, again, significantly reduced in *H. polygyrus* infected mice in this strain (see Figure E1F in the Online Repository).

These findings demonstrate a potent inhibition of RSV-induced disease, early pro-inflammatory cytokine production and recruitment of a broad range of immune cells to the lung in *H. polygyrus* co-infection presumably due to an early reduction in viral infection.

Adaptive immune responses, including Th2 responses, are not required for *H. polygyrus*-induced protection against RSV infection.

Type 2 immune responses are crucial during most helminth infections, aiding in wound healing and immunity to helminths (29-32). IL-4Rα-deficient mice cannot respond to IL-4 or IL-13 signals, and present strongly diminished type 2 immune responses (33). Consistent reductions in RSV titres were observed in *H. polygyrus* co-infected *IL-4Rα*$^{-/-}$ mice, similar to those seen in wild type BALB/c mice (see Figure 2A). We further assessed innate type 2 immune responses, and found that following *H. polygyrus* infection, a non-significant trend for increased IL-13 producing ILC2s and IL-33 levels were observed in the lung tissue, compared to RSV and UV-RSV infected controls (see Figure E2A and E2B in the Online Repository). To investigate any protective role of IL-33 in response to RSV infection, we used *IL-33R*$^{-/-}$ mice. The RSV load was similar between *IL-33R*$^{-/-}$ and wild type control mice (see Figure E2C in the Online Repository), and was reduced to similar levels in both groups by *H. polygyrus* co-infection, indicating that there is no essential role for IL-33 in protection against RSV infection.

To determine if any adaptive immune responses are required for *H. polygyrus*-mediated protection against RSV infection we used RAG1-deficient mice, which lack all T and B cells. Once again, RSV titres were significantly suppressed in both *RAG1*$^{-/-}$ mice and wild type

controls following co-infection with *H. polygyrus* (see Figure 2B). Together, these observations show that adaptive immune responses and IL-4Rα-dependent or IL-33R-dependent type 2 cytokine responses are not required for the protective effect of *H. polygyrus* on RSV infection.

H. polygyrus infection induces expression of type I IFN and IFN stimulated genes in both the duodenum and the lung.

Type I IFNs are major players in the initial response to viral entry into the mucosa (34). Since adaptive and innate type 2 immune responses were not essential for the protection against RSV infection, we hypothesised that *H. polygyrus* enhances the mucosal innate IFN response conferring an antiviral state. *2'5' oligoadenylate synthetase* (*Oas*) and *viperin* are two of many IFN stimulated genes (ISG) which have been found to play a protective role in RSV infection and can be driven by type I IFN signalling (35-38). Gene expression of *IFN*-β, *viperin*, and *OAS1a* tended to increase in the duodenum from day 3 post-*H. polygyrus* infection (see Figure E3 in the Online Repository). Importantly, expression of these genes was also subsequently increased in the lung (see Figure 3A-C), despite the strictly enteric nature of *H. polygyrus*, and remained increased 1 hr after RSV infection (see Figure 3D-F), if this was preceded by *H. polygyrus*. By 6-12 hours after RSV infection, IFN-β transcripts reached the same levels in RSV mono- and co-infected mice (see Figure 3G and 3H). IFN-β protein levels measured by ELISA were below the detection limit at 1 hour after RSV infection and were found at similar levels between groups by 6 hours post-infection reflecting the RTPCR data. However, IFN-α protein was significantly increased at 6 hours after RSV infection (see Figure 3I). This data suggests that pre-existing upregulation of pulmonary type I IFN, *viperin* and *OAS1a*, prior to RSV infection, could underpin *H. polygyrus*-induced protection against RSV infection in the lung.

H. polygyrus-induced protection against RSV infection requires type I IFN receptor signalling.

Since ISG, including viperin and OAS1a, are expressed upon type I IFN receptor signalling, we used *IFNAR1*-deficient mice which fail to signal in response to IFN-α and IFN-β. In *IFNAR1*$^{-/-}$ mice the reduction of RSV load in *H. polygyrus* co-infection was lost, implying an essential role for this pathway in *H. polygyrus* induced protection against RSV infection (see Figure 4A). Furthermore, the ISG induction seen in wild-type mice is also lost in *IFNAR*$^{-/-}$ mice upon *H. polygyrus* infection (see Figure 4B and 4C).

The cathelicidin CRAMP is upregulated during _H. polygyrus_ infection but is not required for expression of type I IFN and ISGs.

Cathelicidins are a family of small, cationic peptides with microbicidal and immunomodulatory properties (39). Humans and mice have only one cathelicidin, LL-37 and mCRAMP respectively, both of which have direct antiviral activity against RSV (40, 41). Cathelicidins have also been shown to promote type I IFN production by DCs (42, 43) and to enhance responses to viral RNA (44). Interestingly, expression of *Camp* (encoding mCRAMP) was also found to be upregulated in both the duodenum and the lung (see Figure E4A and 4B in the Online Repository) during *H. polygyrus* infection, with expression peaking prior to peak type I IFN and ISG expression, and remaining elevated 1 hour after RSV infection (see Figure E4C in the Online Repository). These data suggested that *Camp* expression might be upstream of these responses. Thus, *H. polygyrus* induced type I IFN and ISG expression was investigated in cathelicidin-deficient (*Camp*$^{-/-}$) mice and was found to be intact (see Figure E4D-F in the Online Repository). This indicates that, while potentially contributing to the innate defence against RSV infection, mCRAMP is not the initiator of and

is not required for the protective antiviral immune response induced by *H. polygyrus* infection.

***H. polygyrus* adult excretory secretory products are not responsible for the effects on RSV infection, while larval stages alone confer protection.**

Much interest has been building around the prospect of helminth excretory secretory (ES) products as potential therapeutics (45). *H. polygyrus* ES (HES), secreted by adult worms collected from the intestinal lumen, has been shown to have systemic effects in models of disease, and to mimic the effects of live infection (46). HES was administered in various regimes, by the intranasal and intraperitoneal routes, the day before RSV infection, for a week prior to infection, prior and post-infection and also by continuous HES treatment via an intraperitoneal osmotic mini-pump. None of these protocols resulted in significant reduction in viral titres when compared to RSV infected controls without HES treatment (see Figure E5 in the Online Repository).

The lack of protection afforded by adult worm products, together with the lack of requirement for an adaptive immune response caused us to question whether adult worms play any role in the interaction with RSV, or if larval stages of *H. polygyrus* and the damage associated with their initial invasion of submucosal tissue is key. Therefore, we irradiated stage 3 *H. polygyrus* larvae, as a non-lethal means of preventing their maturation to adulthood (47). The larvae are consequently able to penetrate the duodenal wall and enter into the submucosa, causing the initial trauma associated with infection, but do not re-emerge into the lumen as adults. Irradiated larvae also reduced RSV titres and induced *IFN*-β, *Oas1a* and *viperin* expression (see Figure 5A-D). No adults were found in the lumen in the 300Gy treated group, and numbers were severely reduced following 100Gy irradiation of larvae, but

granulomas were observed in all groups on the duodenal serosa (data not shown), confirming that the irradiated larvae were still able to invade the intestinal mucosal epithelium (48).

The presence of the gut microbiota is essential for *H. polygyrus* induced protection against RSV infection.

Larval stages of *H. polygyrus* protected against RSV infection, and this effect could be attributed to either the direct damage caused upon larval penetration of the submucosa, and/or the consequent translocation of intestinal bacteria into the mucosal tissues. To ascertain whether the microbiota play an important role in protection, we studied RSV infection in germ-free mice in the presence or absence of *H. polygyrus* infection.

In contrast to fully-colonised SPF mice, in germ free mice RSV titres and RSV L gene expression were not supressed by *H. polygyrus* co-infection (see Figure 6A and 6B). Furthermore, the upregulation of type I IFN expression seen in the lung and duodenum of *H. polygyrus* infected SPF mice was absent in *H. polygyrus* infected germ free mice (see Figure 6C and 6D). These data support a model in which the microbiota play a critical role in the induction of type I IFNs and ISGs during *H. polygyrus* infection, which in turn leads to functional antiviral protection in the lung.

DISCUSSION

Here we demonstrate that a strictly enteric helminth can have protective effects against RSV infection in the lung, through a mechanism mediated by microbiota-dependent type I IFN production. Firstly, we established that co-infection with *H. polygyrus* ameliorated RSV-induced disease (manifesting as weight loss and increased respiratory effort) as well as reducing the production of pro-inflammatory cytokines and infiltration of immune cells (NK cells, cDCs, $CD8^+$ T cells and B cells) into the lungs. Unexpectedly, this was associated with, and presumably a consequence of, a reduction in RSV load following *H. polygyrus* co-infection. These protective effects were found to be independent of adaptive immune responses, including Th2 responses, as demonstrated in *RAG*$^{-/-}$ and *IL-4Rα*$^{-/-}$ mice respectively. In addition, these protective effects could not be replicated with HES treatment instead of live infection. Finally, enteric helminth infection upregulated antiviral type I IFN, ISG, and *Camp* gene expression in both the duodenum and the lung, and the protective effects of *H. polygyrus* on RSV infection were dependent on type I IFN receptor signalling and the presence of microbiota, as demonstrated in *IFNAR1*$^{-/-}$ and germ-free mice, which were not protected against RSV infection by *H. polygyrus*.

The role of helminths in co-infections is not well understood (21). In particular, respiratory viral infection in the context of co-infection with helminths has not been investigated in epidemiological studies, nor in any great detail in animal models. *H. polygyrus* co-infection has previously been shown to reduce influenza virus titres and antibodies against the virus regardless of the lifecycle stage of helminth used (24). In addition, *Trichinella spiralis* was found to have protective effects against influenza infection that were dependent on the intestinal phase of infection, enhancing weight gain following influenza-induced weight loss and reducing cellular infiltration into the lung (25). These observations are similar to the

reduced weight loss observed in the *H. polygyrus* and RSV co-infection model reported here, and the reduced cellular infiltrate into the lung. However, the mechanisms involved in this protection were not elucidated in previous studies. More recently, chronic infection with *Schistosoma mansoni* provided significant protection against lethal influenza infection and infection with pneumovirus of mice (PVM) (49). This was found to be dependent upon the presence of eggs, which are known to cause significant damage to the gut wall. *S. mansoni* induced TNF-α dependent induction of Muc5ac and led to goblet cell hyperplasia in the lung, indicating increased epithelial barrier function. However, this was independent of type I IFN production, without any increase in type I IFN in the lung of *S. mansoni* infected mice over controls.

Helminths induce a strong Th2 immune response, which is characterised by high levels of IL-4, IL-5 and IL-13, infiltration of eosinophils, basophils and alternatively activated macrophages, as well as high production of IgE (30-32). In recently reported murine models, helminth induced type 2 immune responses and associated alternative macrophage activation aggravated γ-herpesvirus and norovirus infection (22, 23). However, our data show clearly that the Th2 response is not involved in protection against RSV which was maintained in *IL-4Rα*$^{-/-}$ mice. In fact the helminth-induced adaptive immune response was all together dispensable for protection, indicating an important role for the innate antiviral immune response.

Type I IFNs are an important part of the innate antiviral immune response that can be triggered through activation of pathogen recognition receptors (PRR) by viral components. They not only have direct antiviral activity, but they also have the ability to upregulate the expression of ISGs which have further antiviral potential, thus limiting viral infection and spread. Type I IFNs and ISGs are rapidly upregulated following RSV infection and decline

by 24 hours post infection (50, 51). The ISGs viperin and OAS, have previously been found to play a role in inhibiting RSV infection and have potent antiviral activity (36, 38). In murine models of RSV infection, prior administration of type I IFNs results in a decrease in replication and pathology upon RSV infection (52, 53). In addition, IFN-β treatment has also been shown to have antiviral effects against RSV, through the induction of proteases (54). Administration of recombinant type I IFN in humans has been limited thus far to IFN-α in the context of RSV infection (55-57) and nasal, but not intramuscular, administration prior to RSV challenge reduced signs and symptoms of upper respiratory tract infection (URTI) (56). Administration of recombinant ISGs has not been widely explored, however, RSV infection in chinchillas was reduced after transduction of the airways with vectors encoding viperin (36). However, there is very little evidence linking helminths and type I IFNs in the literature. Aksoy et al, found that double stranded structures found in *S. mansoni* egg RNA triggered TLR3 activation which in turn lead to the activation of the type I IFN response (58). In *H. polygyrus* infection, the type I IFN response has previously been reported to inhibit granuloma formation around larval parasites, but expression of the cytokines in direct response to infection was not measured (59).

H. polygyrus infection induced upregulation of IFN-β transcript, and IFN-α protein levels in the lung at very early (< 6 hour post-infection) time points. This result, combined with the observation that the protective effect of *H. polygyrus* co-infection was lost in IFNAR deficient mice, indicates that upregulation of type I IFN expression by *H. polygyrus* is critical to its antiviral effects. While we were unable to detect IFN-β protein following *H. polygyrus* mono-infection or very early after RSV infection, the extensive gene expression data and in particular the induction of ISGs suggests that *H. polygyrus* induces type I IFN production, at levels too low to be detected by ELISA. We hypothesise that helminth infection and/or

associated bacterial exposure act as a weak signal for cells to produce low levels of type I IFN which through feedback via the IFNAR receptor, induce ISG transcription. This may prime cells to elicit rapid and strong type I IFN and ISG responses upon encounter of a strong stimulus, such as RSV (60). Such priming would benefit the host by enabling the fine balance between necessary rapid efficient anti-viral responses triggered by type I IFNs and detrimental inflammation and autoimmunity associated with chronic type I IFN responses (61). We observed a significant increase in IFN-α protein levels in the lungs of co-infected mice 6 hours post RSV infection. Previous reports indicate that IFN-β is effective in inducing IFN-α production (but not vice versa) (62), therefore we speculate that the early increase in IFN-β production could lead to the observed increase in IFN-α. Based on the recently described central role of alveolar macrophages in the production of type I IFN during RSV infection (63), we speculate that these cell are also the likely source of helminth induced type I IFN in the lung.

Irradiation of stage 3 *H. polygyrus* larvae has been previously shown to inhibit their maturation, but allows larval migration into the intestinal submucosa, after which point the larvae do not develop further into adults (47). By taking this approach, we demonstrated that larval stages are sufficient to induce IFN-β and ISG gene expression and to confer protection against RSV infection. Further investigation in germ free mice revealed a requirement for microbiota in helminth-induced IFN-β upregulation and resistance to viral replication. It is therefore plausible to speculate that the damage caused by initial penetration of larvae into the submucosa may result in bacterial translocation from the gut and activation or priming of the innate immune response. Indeed, upregulation of type I IFNs at epithelial barrier surfaces can reduce bacterial translocation by upregulating tight junctions (64). Thus bacterial translocation in the intestine during *H. polygyrus* infection may induce upregulation of type I

IFNs systemically to limit such translocation. In addition, commensal, but not pathogenic, bacteria have been shown to induce type I IFN production and can also provide protection against influenza infection (65-67). Furthermore, helminth infection has been shown to alter the microbiome in the intestine of both humans and mice. A study conducted in Malaysia indicated that helminth infected people had a greater bacterial number and richer diversity, with increases in specific bacterial taxa, than uninfected controls (68). Likewise in mice, parasites including *Trichuris muris* and *H. polygyrus* have been found to alter the balance of commensals in the intestine (69, 70). A specific increase in *Lactobacillus* species has been noted during *H. polygyrus* infection (70). Interestingly, the administration of *Lactobacillus* species prior to RSV infection, either by intranasal or oral routes, can increase antiviral immunity, including an increase in IFN-β in the bronchoalveolar lavage and therefore resistance to RSV infection (71, 72).

Viral LRTI with RSV and rhinoviruses in the first years of life has been linked to the development of asthma (73-75), which helminth infections have been shown to protect against in mouse model systems (76, 77). In parallel, intestinal helminth infections in humans have been reported to increase bacterial translocation (78). Thus, we speculate that helminth infection may protect against severe respiratory viral infections in early life, and that this effect in turn may contribute to a reduced potential for asthma development.

In conclusion, we show that intestinal helminth infection can be beneficial in respiratory viral infection. Based on our findings we hypothesise that helminth infection in the gut triggers type I IFN production through bacterial interactions, which leads to systemic type I IFN induction thus raising preparedness of remote sites such as the lung to mount an effective innate response against incoming unrelated viral pathogens. In addition to these findings, further work will be required to elucidate the exact mechanisms of *H. polygyrus* induced

469 antiviral effects, and thus inform potential translation towards new helminth-based
470 approaches to prevention and treatment of respiratory viral disease.

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Author Contributions

AJM, HJM, DJD, RMM, JS contributed to experimental conception, design, data analysis and interpretation. AJM, HJM, PMF, CW, KJM, ESG, CJCJ performed experiments. DJD, ASM, MRE, NLH, BJM, RMM contributed essential reagents or tools. AJM, HJM, RMM, DJD and JS contributed to manuscript preparation.

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682 **Table-I: Primers used for Real-time PCR**

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (FAM-TAMRA 5'-3')
OAS-1a	TCCTGGGTCATGTTAATACTTCCA	GAGAGGGCTGTGGTGGAGAA	CAAGCCTGATCCCAGAATCTATGCC
Viperin	CGAAGACATGAATGAACACATCAA	AATTAGGAGGCACTGGAAAACCT	CCAGCGCACAGGGCTCAGGG
RSV-L	GAACTCAGTGTAGGTAGAATGTTTGCA	TTTCAGCTATCATTTTCTCTGCCAAT	TTTGAACCTGTCTGAACATTCCCGGTT

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Figure legends

Figure 1. *H. polygyrus* infection attenuates RSV disease and inflammation and reduces RSV viral load.

The standard co-infection protocol was used as follows: female BALB/c mice were given 200 *H. polygyrus* L3 larvae by oral gavage at day -10 or left naive. At day 0, $6x10^5$ PFU (A, B) or $4x10^5$ PFU (C, D, E) RSV or UV-inactivated RSV was administered intranasally, (**A**) Mice were weighed daily and percentage of original weight is shown; (**B**) Enhanced pause (penH) was assessed by whole body plethysmography (WBP); (**C, D**) Samples were taken at the indicated time points after RSV infection for flow cytometric analysis. Numbers of $CD3^+$ $CD8^+$ T cells **(C)** and of $MHCII^+CD11b^+CD11c^+$ conventional dendritic cells **(D)** per right lung lobe are shown; (**E**) Lungs were harvested on days 3, 4 and 6 post RSV infection and plaque assays performed. All data are depicted as mean ± SEM. Data in A & B pooled from 2 independent experiments, total n=8 per group, in C, D & E from 2 independent experiments, total n=6 per group per time point. Statistical significance of differences between RSV infected groups was determined by two-way ANOVA with Bonferroni's post hoc test. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Figure 2. Adaptive immune responses, including Th2 responses, are not required for the *H. polygyrus*-mediated attenuation of RSV viral titres.

The standard co-infection protocol was followed **(A)** in BALB/c IL-4Rα deficient mice and **(B)** in BALB/c RAG1 deficient mice. Lungs were harvested on day 4 of RSV infection and plaque assays performed to determine RSV titres**.** All data are depicted as mean ± SEM. Data in A are pooled from 2 individual experiments, total n=4-8 per group. Data in B are representative of 2 independent experiments, n=3-4 per group. Statistical significance

between groups was determined by one-way ANOVA with Tukey's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$.

Figure 3. *H. polygyrus* induces type I IFN and associated gene expression in the lung. BALB/c **(A-C)** were given 200 L3 *H. polygyrus* larvae or left naïve. At indicated time-points post-*H. polygyrus* infection half of the large left lung lobe was placed in Trizol and RTPCR was performed for expression levels of **(A)** *IFN*-β, **(B)** *OAS1a* or **(C)** *viperin* in lung comparing *H. polygyrus* infected to naïve mice. The standard co-infection protocol was followed in BALB/c mice (**D-I**). 1 hour after RSV infection half of the large left lung lobe was placed in Trizol and RTPCR was performed for expression levels of (**D**) *IFN*-β (**E**) *OAS1a* (**F**) v*iperin*. **(G-I)** 1 (data from Fig. 3A), 6 and 12 hours post-RSV infection half of the large left lung lobe was placed in **(G)** Trizol and RTPCR was performed for expression levels of *IFN*-β; **(H&I)** was homogenized and **(H)** IFN-β and **(I)** IFN-α protein levels were analysed by ELISA. (A-G) results were normalised to *18S* expression and represented as fold change in expression over naïve controls (A-C), UV-RSV controls (D-G). Data are depicted as mean ± SEM. Data are pooled in A-I from 2 independent experiments, total n=6-8 per group and in I from 2 individual experiments, total n=10 per group. Statistical significance of differences between groups was determined, A-C by one-way ANOVA with Bonferroni's post hoc test and in D-I by two-way ANOVA with Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, NS = non-significant.

Figure 4. Type I IFN signalling is essential for *H. polygyrus*-induced protection against RSV.

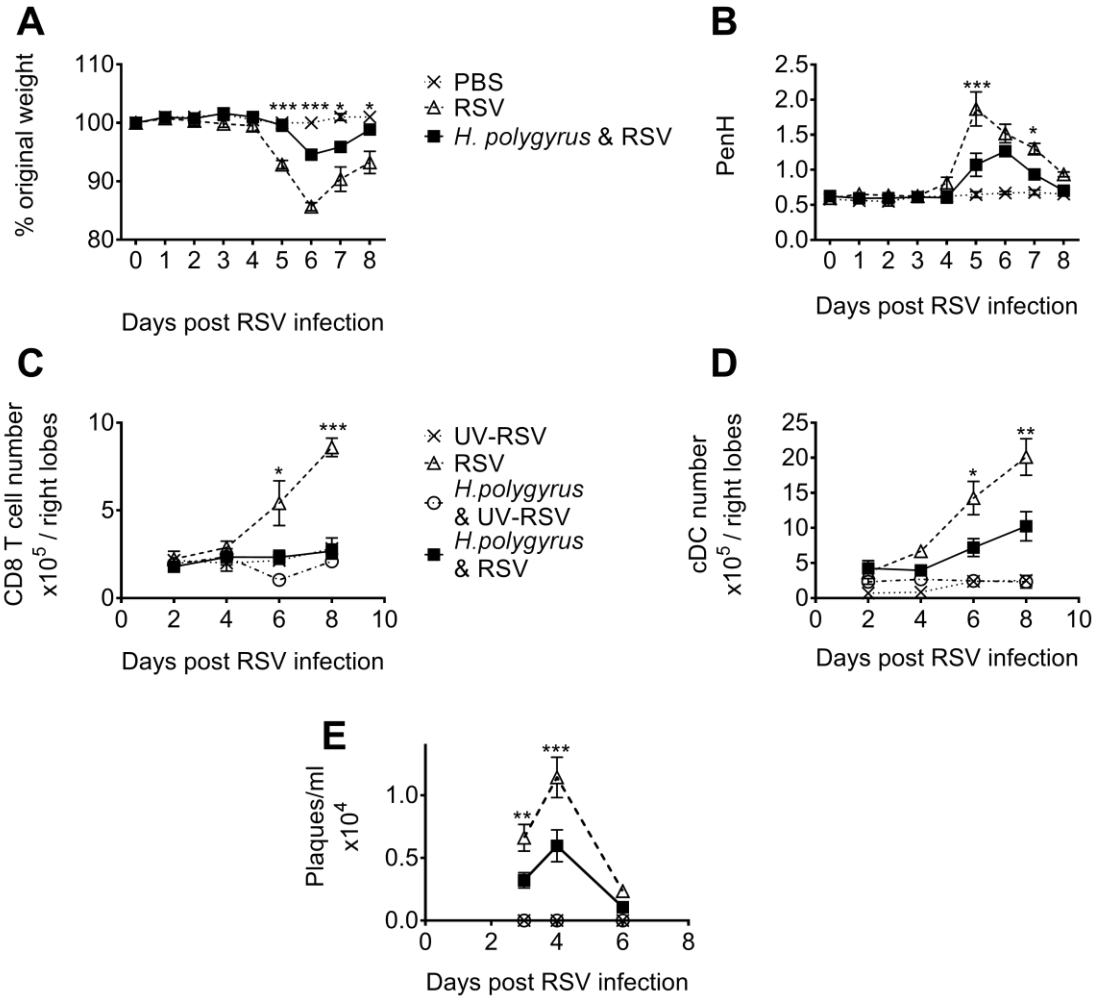
(A) The standard co-infection protocol was followed in C57BL/6 or IFNAR1 deficient mice or were given 200 L3 *H. polygyrus* larvae or left naïve. 3 days post-RSV infection half of the

large left lung lobe was placed in Trizol and RTPCR was performed for expression of RSV L gene. **(B&C)** 10 post-*H. polygyrus* infection half of the large left lung lobe was placed in Trizol and RTPCR was performed for expression levels of **(B)** *OAS1a* or **(C)** *viperin* in lung comparing *H. polygyrus* infected to naïve mice. All results were normalised to *18S* expression and represented as fold change in expression over naïve/RSV controls. Data are depicted as mean ± SEM. Data are pooled from 2 independent experiments, total n=6-10 per group. Statistical significance of differences between groups was determined by two-way ANOVA with Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, NS = non-significant.

Figure 5. *H. polygyrus* larval stages are sufficient to protect against RSV infection.
200 L3 *H. polygyrus* larvae were irradiated at 300 Gy and compared to non-irradiated larvae in **(A)** standard co-infection protocol; or **(B-D)** to naïve controls following *H. polygyrus* infection alone. **(A)** Lungs were harvested on day 4 of RSV infection and plaque assays performed. **(B-D)** On day 10 of *H. polygyrus* infection the right lung lobes were removed and placed in Trizol for RTPCR for *IFN*-β, *OAS1a* and *viperin*. All results were normalised to *18S* expression and represented as fold change in expression over controls. All data are depicted as mean ± SEM. A-D is representative of two individual experiments, total n=3-4 per group. Statistical significance of differences between groups was determined in (A) by one-way ANOVA with Tukey's post hoc test and (B) by unpaired t-test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, NS= non-significant.

Figure 6. Microbiota are required to protect against RSV infection.
The standard co-infection protocol was followed in BALB/c germ-free and SPF mice, using 400L3 germ free *H. polygyrus* larvae and $3x10^7$ sterile RSV in 100µl. On day 4 after RSV

infection, **(A)** the left lung lobe was removed and plaque assays performed; **(B)** the right lung lobes were removed and placed in Trizol for RTPCR for RSV L gene or **(C)** *IFN*-β expression; **(D)** the first centimetre of the duodenum was removed and placed in Trizol and RTPCR was performed for expression of *IFN*-β. Results in B-D are normalised to *18S* and represented as fold change in expression over SPF RSV infected controls. All data are depicted as mean ± SEM. All data are representative of two individual experiments, total n=3-4 per group. Statistical significance of differences between groups was determined by unpaired t-test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, NS= non-significant.



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770 Figure 1

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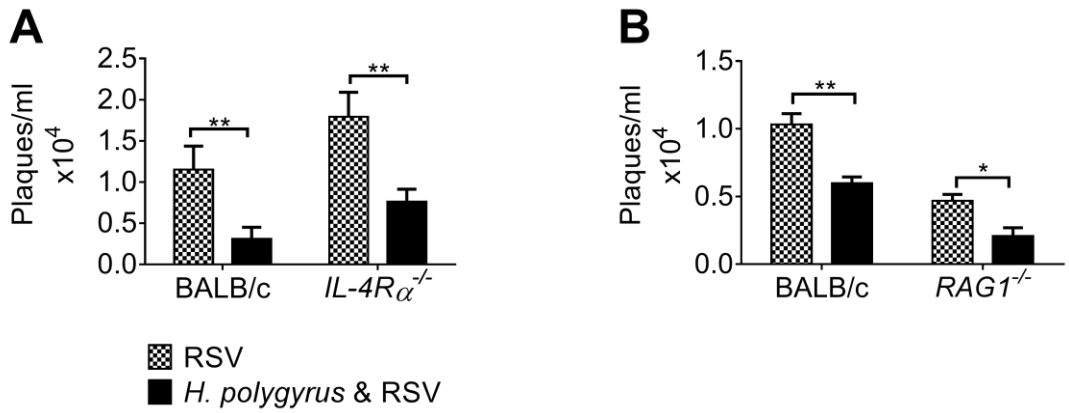

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773 Figure 2

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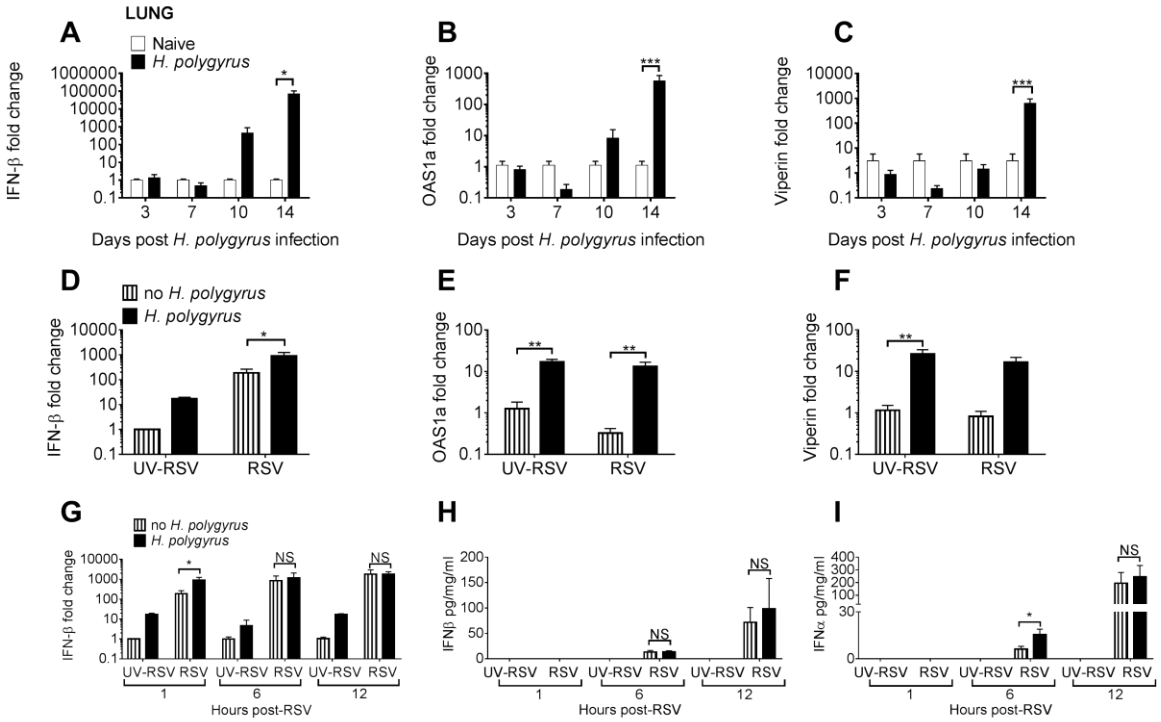

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776 Figure 3

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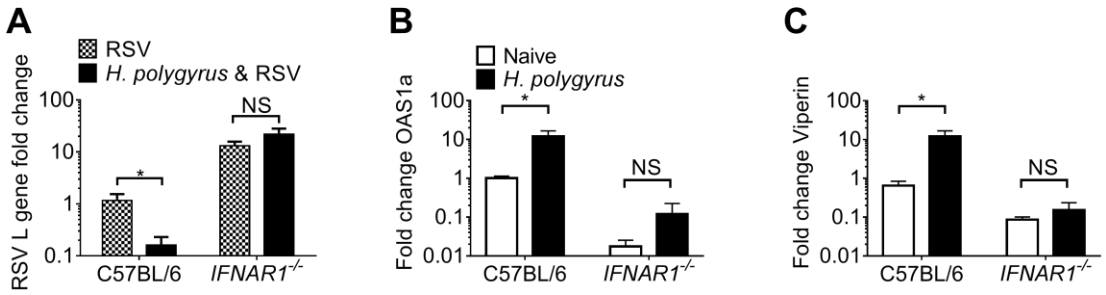

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779 Figure 4

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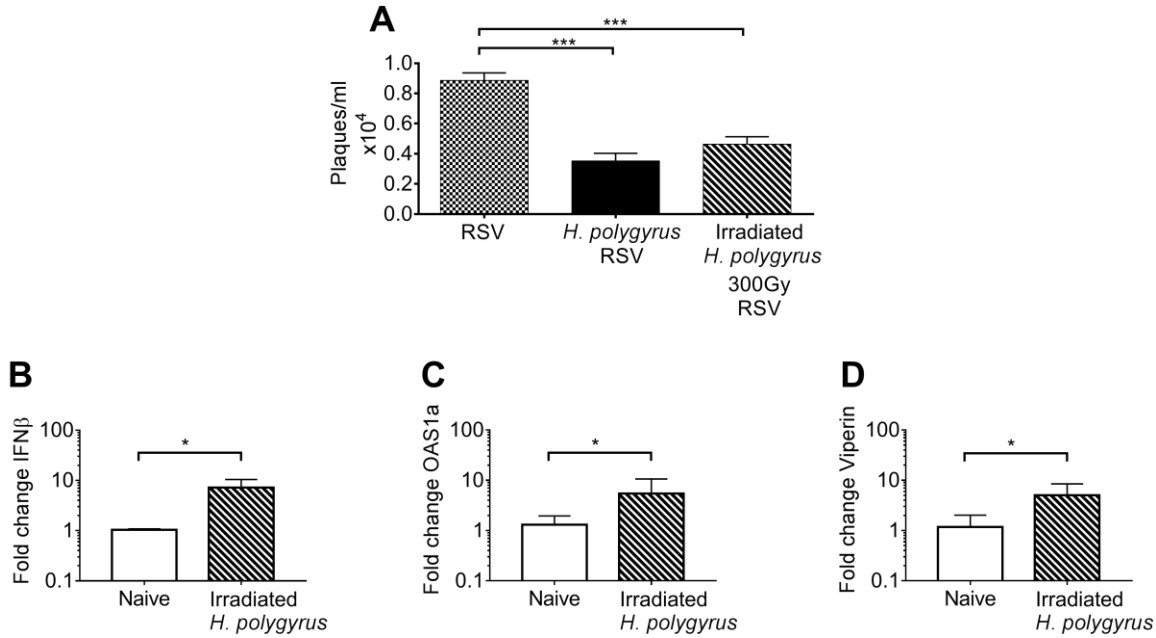

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782 Figure 5

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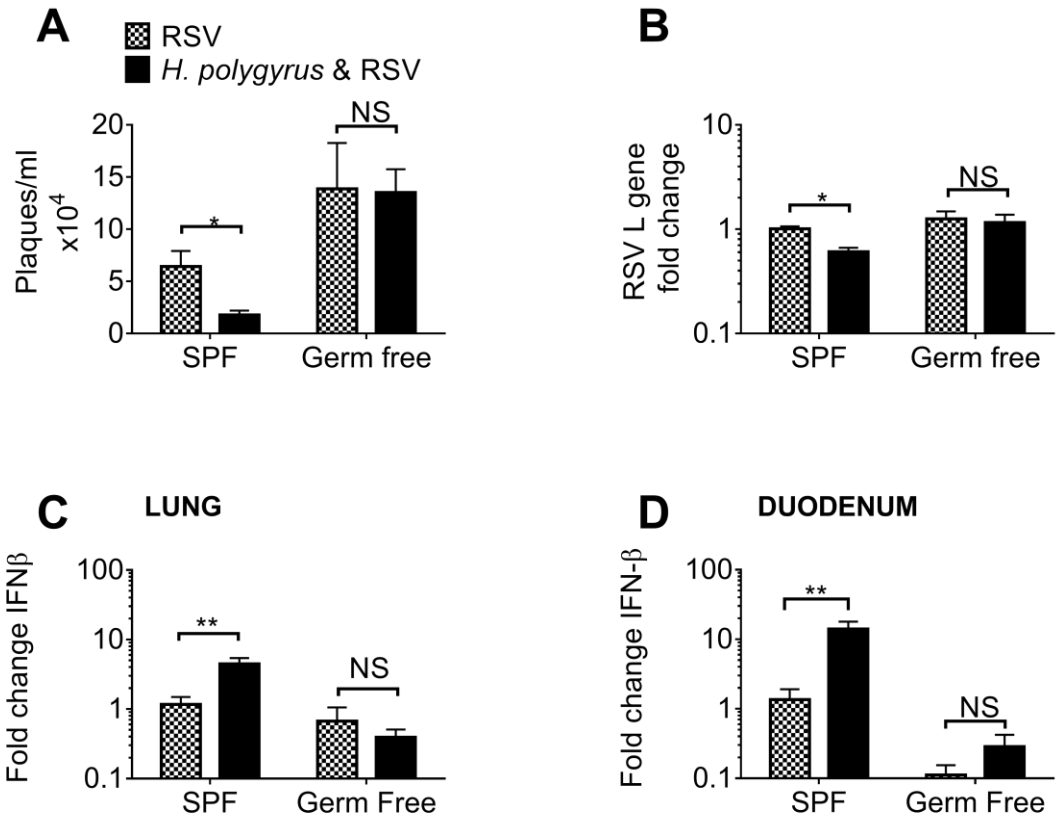

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785 Figure 6

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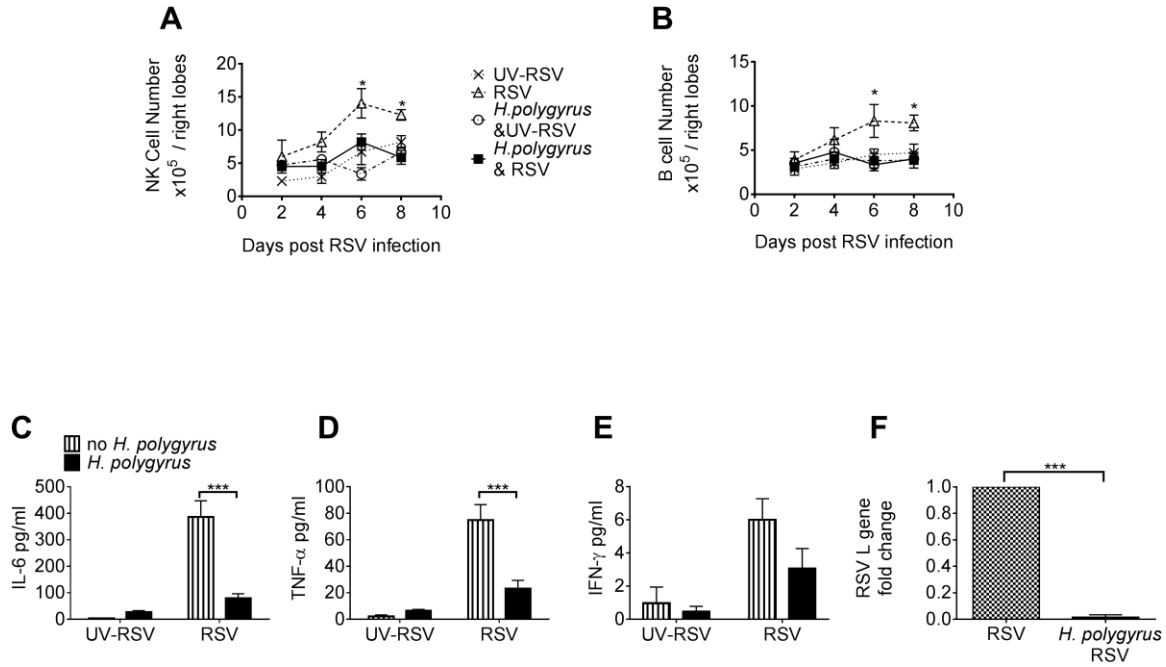

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788 Figure E1

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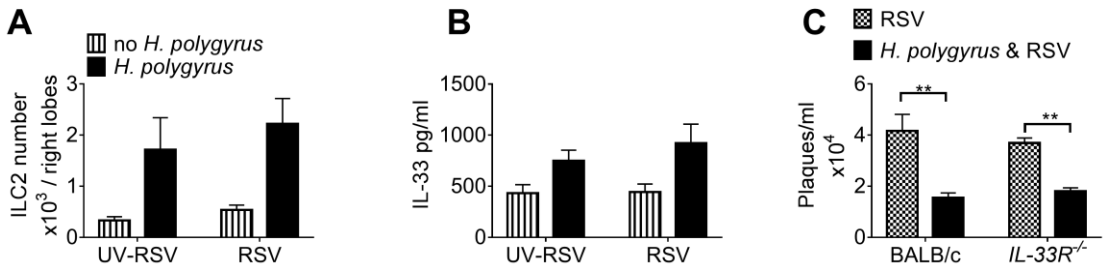

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791 Figure E2

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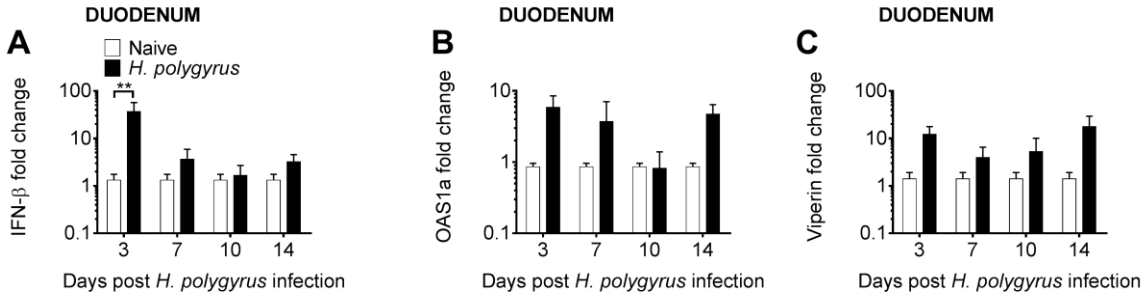

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794 Figure E3

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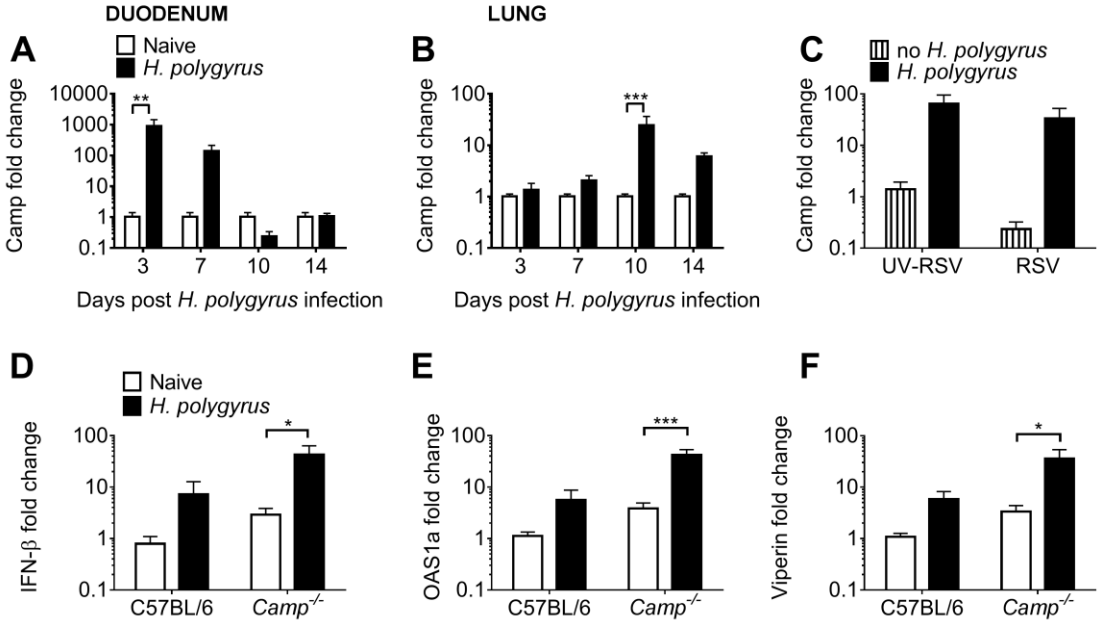

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797 Figure E4

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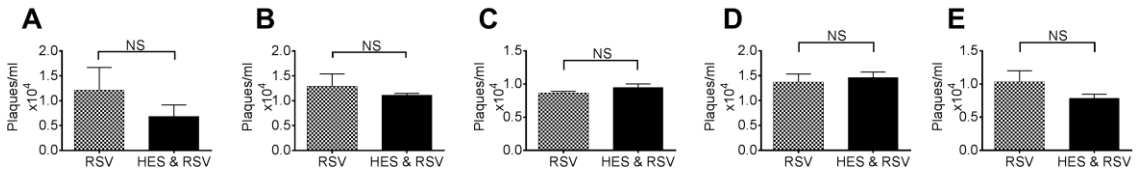

Figure E5

ONLINE REPOSITORY

TITLE PAGE

Original Article

Enteric helminth-induced type-I interferon signalling protects against pulmonary virus infection through interaction with the microbiota

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METHODS

Cytometric Bead Array

Half of the left lung lobe was homogenised using a TissueLyser (Qiagen) in 0.5ml of 1x cell lysis buffer (Cell Signalling, Danvers, MA, USA) containing 1µg PMSF (Sigma). Cytokines present in the lung homogenate were detected through the use of a Cytokine Bead Array flex set (BD Biosciences), following the manufacturer's protocol was followed. Samples were collected on the FACS Array (BD) and analyzed using Flowjo software (version 7.6.5).

ELISA

IL-33 was measured using the R&D Systems ELISA kit according to the manufacturer's instructions.

Osmotic Minipump Surgery

Minipumps (Alzet, Cupertino, CA) were filled with the appropriate volume and concentration of HES prior to implantation, and primed in saline at 37°C overnight. Mice were placed under general anaesthesia using inhalable isoflurane and were given 0.1mg/kg subcutaneous buprenorphine. The peritoneal cavity area was shaved and the area was swabbed with alcohol to provide a sterile environment. A midline incision was made just below the ribcage, about 1cm in length. The musculoperitoneal layer was lifted using forceps to avoid internal damage, and an incision was made in the peritoneal wall beneath. The primed minipump was then inserted into the cavity, with the delivery port entering first, and the wound was then closed using interrupted sutures. Mice were monitored upon recovery from anaesthetic, and were given a further 0.1mg/kg subcutaneous buprenorphine post-op

Figure legends

Figure E1: *H. polygyrus* infection attenuates RSV inflammation and reduces viral load.

The standard co-infection protocol was followed. Samples were taken at the indicated time points after RSV infection for flow cytometric analysis. **(A)** Total number of $CD49B^+NKP46^+$ NK cells per right lung lobe; **(B)** Total number $MHCII^+CD19^+B220^+$ B cells per right lung lobe; **(C, D)** Half of the left lung lobe was homogenized and cytokine levels were analysed by Cytometric Bead Array (CBA) levels of **(C)** IL-6 and **(D)** TNF-α **(E)** IFN-γ levels were determined. (**F**) The standard co-infection protocol was followed in female C57BL/6 mice. 3 days post-RSV infection half of the large left lung lobe was placed in Trizol and RT-PCR was performed for expression of the RSV L gene. All data are depicted as mean ± SEM and are pooled from 2 independent experiments, total n=6 per group per time point. Statistical significance of differences between RSV infected groups were determined, in A-E by two-way ANOVA with Bonferroni's post hoc test and in F by unpaired t-test. $^{*}P<0.05$, $^{***}P<0.001$.

Figure E2: IL-33 is not essential for protection against RSV.

The standard co-infection protocol was followed in **(A & B)** BALB/c or in **(C)** BALB/c *IL-33R*$^{-/-}$ mice. Samples were taken 1 hour after RSV infection **(A)** for flow cytometric analysis of numbers of $ICOS^+$ IL-13^+ ILCs per right lung lobe; **(B)** half of the left lung lobe was homogenized and cytokine levels were analysed by ELISA; **(C)** The standard co-infection protocol was followed in BALB/c *IL-33R*$^{-/-}$ mice. Lungs were harvested on day 4 of RSV infection and plaque assays performed to determine titres. All data are depicted as mean ±

SEM. Data are pooled from 2 independent experiments, total n=6-8 per group. Statistical significance of differences between groups was determined by two-way ANOVA with Bonferroni's post hoc test. *P<0.05, **P<0.01, ***P<0.001.

Figure E3: *H. polygyrus* induces type I IFN and associated gene expression in the intestine.

BALB/c mice were given 200 L3 *H. polygyrus* larvae or left naïve. The 1st cm of the duodenum was placed in Trizol and RT-PCR was performed for expression levels of *IFN*-β, *OAS1a, Viperin* **(A-C)** comparing *H. polygyrus* infected to naïve mice. Results were normalised to *18S* expression and represented as fold change in expression over naïve controls. Data are depicted as mean ± SEM. Data are pooled from 2 independent experiments, total n=6-8 per group. Statistical significance of differences between groups was determined by one-way ANOVA with Bonferroni's post hoc test. $^{**}P<0.01$

Figure E4: *Camp* does not drive type I IFN and ISG expression in the lung.

BALB/c mice were given 200 L3 *H. polygyrus* larvae or left naïve. **(A)** The 1st cm of the duodenum and **(B)** half of the large left lung lobe was placed in Trizol and RT-PCR was performed for expression levels of *Camp* comparing *H. polygyrus* infected to naïve mice. **(C)** The standard co-infection protocol was followed in BALB/c mice and 1 hour after RSV infection half of the large left lung lobe was placed in Trizol and RT-PCR was performed for expression levels of *Camp*. **(D-F)** C57BL/6 or $Camp^{-/-}$ mice were given 200L3 *H. polygyrus* by oral gavage or left naïve. Half of the large left lung lobe was placed in Trizol and RT-PCR was performed for expression levels of (**D**) *IFN*-β, (**E**) *OAS1a*, and (**F**) *Viperin*. All results

were normalised to *18S* expression and represented as fold change in expression over naïve controls (A & B), UV-RSV controls (C), or C57BL/6 naive controls (D-F). Data are depicted as mean ± SEM. Data are pooled from 2 independent experiments, total n=6-8 per group. Statistical significance of differences between groups was determined in A & B by one-way ANOVA with Bonferonni's post-hoc test and in C-F by two-way ANOVA with Bonferroni's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Figure E5: RSV titres are not inhibited by HES administration.

5μg HES was given to mice (**A**) intranasally on day -1 & 0, (**B**) intranasally on day -7, -4, -1 & 0, (**C**) intraperitoneally on day -7, -4, & -1. Osmotic minipumps containing HES were surgically implanted on (**D**) day -7, (**E**) day -10, releasing 0.25 μl HES per hour for 10 or 14 days respectively. On day 0 $4x10^5$ PFU RSV was administered intranasally. Lungs were harvested on day 4 of RSV infection and plaque assays performed. All data are depicted as mean ± SEM, total n=4 per group. Statistical significance of differences between groups was determined by unpaired t-test. NS= non-significant.